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The Wnt–β-catenin signaling pathway has been shown to govern T cell development by regulating the growth and survival of progenitor T cells and immature thymocytes. We explore the role of noncanonical, Wnt–Ca^{2+} signaling in fetal T cell development by analyzing mice deficient for Wnt5a. Our findings reveal that Wnt5a produced in the thymic stromal epithelium does not alter the development of progenitor thymocytes, but regulates the survival of αβ lineage thymocytes. Loss of Wnt5a down-regulates Bax expression, promotes Bcl-2 expression, and inhibits apoptosis of $CD4^+CD8^+$ thymocytes, whereas exogenous Wnt5a increases apoptosis of fetal thymocytes in culture. Furthermore, Wnt5a overexpression increases apoptosis in T cells in vitro and increases protein kinase C (PKC) and calmodulin-dependent kinase II (CamKII) activity while inhibiting β-catenin expression and activity. Conversely, Wnt5a deficiency results in the inhibition of PKC activation, decreased CamKII activity, and elevation of β-catenin amounts in thymocytes. These results indicate that Wnt5a induction of the noncanonical Wnt–Ca^{2+} pathway alters canonical Wnt signaling and is critical for normal T cell development.

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Members of the Wnt family of secreted glycoproteins function as critical regulators of cell fate and cell growth during development. To date, 20 different mammalian Wnt genes have been identified and categorized based upon their ability to activate different signaling pathways in the cell and to transform cells in culture (1–4). The canonical-signaling mammalian Wnts (typified by Wnt1, 3a, and 8) bind to a cell surface receptor complex composed of the appropriate member of the Frizzled (Frz) family of seven transmembrane proteins and the low-density lipoprotein receptor-related LRP-5 or -6 (5–7). This binding activates the cytosolic phosphoprotein Disheveled and inhibits the function of a degradation complex containing the adenomatous polyposis coli protein Axin and glycogen synthase kinase 3. Inhibition of glycogen synthase kinase 3, which phosphorylates β-catenin and targets it for ubiquitin-mediated proteosomal degradation, leads to the accumulation of β-catenin and transport into the nucleus by Pygopus and legless/BCL-9. Nuclear β-catenin complexes with the transcription factors LEF/TCF to transform these proteins from transcriptional repressors into activators (8). Thus, canonical Wnt signaling increases β-catenin stability and up-regulates the expression of LEF/TCF-induced genes whose products regulate cell proliferation and differentiation. Inappropriate activation of human canonical Wnt signaling has often been associated with cell transformation in vitro and with several types of human cancer (4, 9).

In contrast, noncanonical Wnt signaling involves recognition of extracellular Wnt ligands by a cognate Frz–LRP receptor complex, heterotrimeric G protein activation of phospholipase C and turnover of phospholipid membranes in the endoplasmic reticulum, and the release of intracellular calcium ions. In some contexts, increased intracellular Ca^{2+} activates protein kinase C (PKC), as well as other calcium-sensitive enzymes, such as calmodulin-dependent kinase II (CamKII), calcineurin, and the calcineurin-dependent, NFAT transcription factor (10). Although transduction of cells with noncanonical WNT ligands such as Wnt5a only weakly transforms cells in vitro (4), activation of the Wnt–Ca^{2+} pathway by Wnt5a has been found to up-regulate the proliferation of mesenchymal cells during mouse axial formation

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and limb development (11), regulate convergent extension movements of *Xenopus laevis* and zebrafish embryos (12–15), and to govern cell planar polarity in vertebrates and *Drosophila melanogaster* (16). Furthermore, activation of the Wnt–Ca^{2+} signaling pathway by Wnt5a has been demonstrated to inhibit β-catenin stabilization and antagonize the canonical Wnt–β-catenin signaling pathway in vertebrate axis formation and limb development in zebrafish and mice, respectively (17, 18). More recently, treatment of HEK293 cells with WNT5a ligand was found to induce β-catenin stabilization and up-regulate TCF/LEF promoter activity, as well as to inhibit canonical Wnt signaling without altering β-catenin levels, depending on the WNT receptor expressed on the cell (19). Therefore, Wnt5a is likely to have multiple, and sometimes opposing, roles in cell growth and development, depending on which signaling pathways are induced or suppressed in the cell.

Canonical Wnt signaling has been proposed to play important roles in the development of mammalian T cells (20). T cells develop primarily in the thymus, where precursor cells undergo distinct proliferation and differentiation steps that are tightly regulated by the thymic stromal microenvironment. Precursor T cells lacking CD4 and CD8 undergo TCR β-chain rearrangement and begin to proliferate and express CD4 and CD8 coreceptors on the cell surface. These immature $CD4^+CD8^+$ double-positive (DP) cells will, in turn, generate mature single-positive (SP), self-MHC–restricted $CD4^+$ or $CD8^+$ T cells, which then leave the thymus and enter the peripheral circulation. Several different molecular pathways, including Wnt signaling, coordinate the development of T cells in the thymus (21). Two major T cell types expressing different TCRs are generated in the thymus: conventional $\alpha\beta TCR^+$ T cells of adaptive immunity and $\gamma\delta TCR^+$ T cells that exhibit traits of both adaptive and innate immunity. T cells of the αβ lineage undergo a well-defined developmental progression, with each stage distinguished by the cell surface antigens CD4 and CD8. Double-negative (DN) $CD4^-CD8^-$ precursor cells, consisting of four subsets (DN1–DN4) defined by distinct CD25 and CD44 expression patterns, differentiate into immature $CD4^+CD8^+$ DP thymocytes if they express functional TCR. A minor subset of DP cells can then be selected based on their TCR specificity and differentiate into either helper $CD4^+CD8^-$ or cytotoxic $CD4^-CD8^+$ mature SP thymocytes. Developmental steps in the γδ lineage are not well characterized, but these cells arise early from the DN stage, and most do not express CD4 or CD8.

Numerous Wnt genes are expressed in thymocytes or in thymic epithelial stromal cells (22), and overexpression of Wnt genes can increase thymocyte proliferation in vitro (23). Mice deficient in LEF1/TCF1 are depleted of αβ lineage thymocytes, and overexpression of extracellular Wnt inhibitors can partially block αβ T cell development in fetal thymic organ cultures (FTOCs) (24–26), suggesting that canonical Wnt signaling is essential for normal thymocyte development. In addition, deletion of Wnt1 and Wnt4 in mice reduces the number of thymocytes by ∼50% compared with normal mice (27), and soluble Frz receptors can block thymocyte development in FTOCs at the DN–DP transition (23). In contrast, where examined, γδ T cells appear largely unaffected by the manipulations of WNT signaling (21). Collectively, the data suggest that canonical WNT signaling is primarily critical in regulating proliferation and survival of αβ T cell lineage at the DN–DP stages. Although the rescue of thymopoiesis in TCF1-deficient mice by expression of TCF1 transgenes has been found to require the β-catenin–interacting region of TCF1 (28), mice deficient in β-catenin display unexpectedly limited alterations in T cell development (29), raising the question as to the extent to which TCF1 function is controlled by β-catenin in thymocytes. Regardless of the precise mechanism, the importance of Wnt–β-catenin signaling in thymopoiesis is widely accepted. However, nothing is known about the function of noncanonical Wnt–Ca^{2+} signaling in thymocyte development.

Several lines of evidence indicate that Wnt5a is an important regulator of cell growth in hematopoietic tissue, and its expression in the thymus raises the possibility for the existence

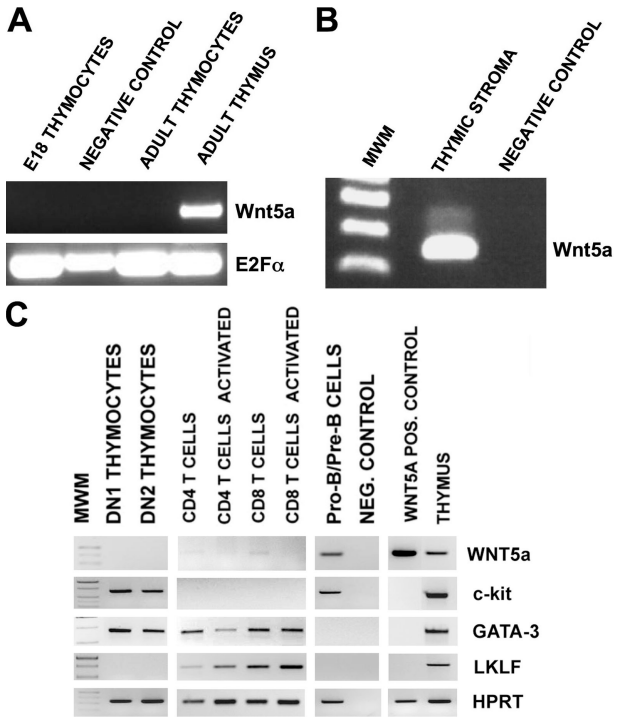

Figure 1. Expression pattern of Wnt5a in thymus and thymocytes. (A) RT-PCR analysis of Wnt5a expression in fetal and adult thymocytes or thymus. PCR of total RNA isolated from adult thymocytes in the absence of RT served as the negative control. (B) RT-PCR analysis of Wnt5a expression in stroma of E18 WT mice. PCR of total RNA isolated from the stroma in the absence of RT served as the negative control. (C) RT-PCR analysis of Wnt5a expression in FACS-purified DN1 ($CD44^+CD25^-$) thymocytes, DN2 ($CD44^+CD25^+$) thymocytes, SP $CD4^+$ and $CD8^+$ thymocytes, BM-derived pro-/pre-B cells ($B220^+CD43^-$ and $B220^+CD43^+$ cells), and HTY cells overexpressing Wnt5a and thymus as positive controls. Amplification of c-kit receptor, transcription factor GATA-3, and the housekeeping gene HPRT served as controls for sorted cell populations

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of noncanonical WNT signaling during T cell development. Addition of Wnt5a to cultured cells stimulates lymphoid and myeloid progenitor cells, and intraperitoneal injection of Wnt5a in mice was found to augment the repopulating capacity of transplanted human hematopoietic stem cells (30). In addition, we have shown that Wnt5a signals through the noncanonical Wnt–Ca^{2+} pathway to inhibit B cell proliferation and spontaneous B cell tumorigenesis in mice (31). To determine if Wnt5a also plays a role in T cell development, we examined T cell development and apoptosis in fetal mice deficient for Wnt5a and in FTOCs derived from these mice. Our results reveal that noncanonical Wnt–Ca^{2+} signaling induced by Wnt5a originating in the thymic epithelium operates in the thymus to govern survival of DP and mature SP thymocytes in a manner antagonistic to canonical WNT signaling.

RESULTS AND DISCUSSION

Previous experiments using RT-PCR assays have documented the expression of multiple Wnt genes in mouse thymic epithelium, including Wnt1, 4, 5b, 7a, 7b, 10a, and 10b (21, 24). Less is known about the expression of noncanonical Wnt genes in mouse thymus, although expression of WNT5a has been detected previously in human thymic stroma (21). To determine if Wnt5a is expressed in mouse thymocytes or stromal epithelial cells, total RNA was extracted from C57BL/6 mouse thymi or from epithelial cells cultured from mouse thymi. Wnt5a expression was readily detected in thymi isolated from perinatal (embryonic day [E] 18) and adult mice by RT-PCR, but was not detected in thymocytes isolated from E18 or adult mice (Fig. 1 A). However, Wnt5a expression was observed in stromal cells derived from newborn mice (Fig. 1 B). Defined adult thymocyte subsets were purified from C57BL/6 mice using fluorescence-activated cell sorting. RT-PCR analysis of Wnt5a expression was performed in parallel with control RT-PCR assays for c-Kit (precursor-restricted), Gata-3 (T cell lineage-specific), or Hprt (positive control) expression to confirm the identity of the sorted T cell populations (30, 31). In some samples, CD4 and CD8 cells isolated from C57BL/6 mice were stimulated for 3 d with immobilized anti-CD3 (5 μg/ml) and anti-CD28 (0.1 μg/ml) to activate T cells. The results (Fig. 1 C) indicate that Wnt5a is expressed at barely detectable levels in mouse SP thymocytes, but Wnt5a expression is not detected in DN1 ($CD44^+CD25^-$) and DN2 ($CD44^+CD25^+$) precursor subsets. These results indicate that Wnt5a is produced primarily by the thymic epithelium during T cell development.

Mice lacking Wnt5a die at birth and display severe caudal defects and reduced morphogenesis of all tissues that outgrow from the main body axis (11). Staining of thymocytes harvested from E17.5 $Wnt5a^{-/-}$ embryos (C57BL/6 background) revealed no difference in the percentage of $CD25^+$, $CD44^+$, and $CD25^+/CD44^+$ cells, indicating normal DN precursor differentiation (unpublished data). To explore a role for Wnt5a signaling in T cell development, FTOCs were

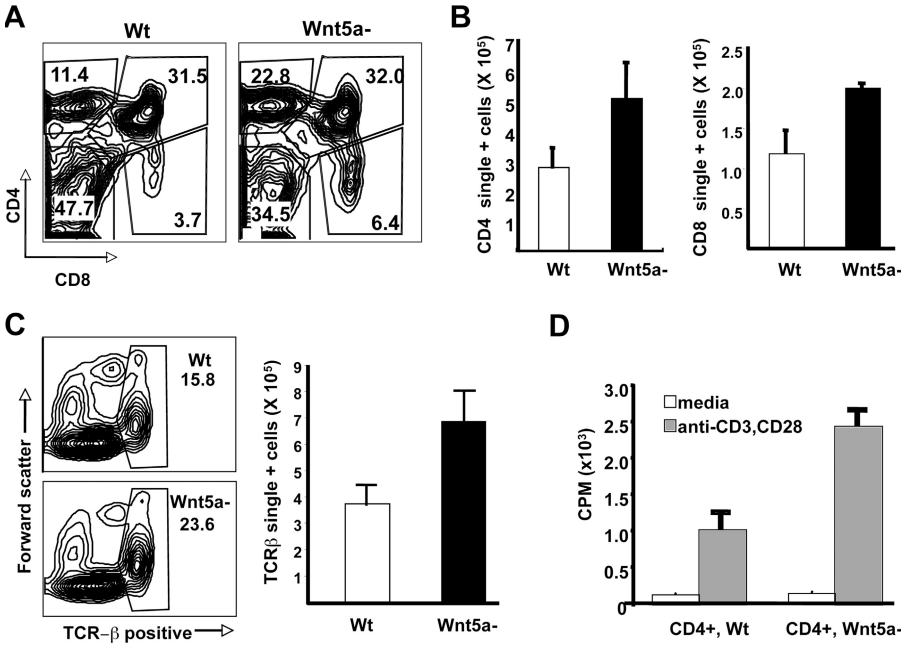

Figure 2. Differentiation and hyperproliferation of thymocytes in $Wnt5a^{-/-}$ mice. (A) Representative result of flow cytometry analysis of CD4 and CD8 antibody-stained $Wnt5a^{+/+}$ and $Wnt5a^{-/-}$ FTOC cells. (B) Mean total numbers of $CD4^+$ or $CD8^+$ cells in FTOCs derived from four embryos for each genotype. (C) Representative flow cytometry analysis of TCR-β antibody-stained $Wnt5a^{+/+}$ and $Wnt5a^{-/-}$ FTOC cells, and mean percentage of TCR-β SP cells in FTOCs derived from four embryos given for each genotype. (D) [^{3}H]thymidine uptake assay performed on cultured $CD4^+$ cells, sorted from $Wnt5a^{+/+}$ and $Wnt5a^{-/-}$ FTOCs in the presence or absence of anti-CD3 and -CD28 antibodies. All experiments presented in this figure were performed using three samples, and were repeated three times with similar qualitative results. Error bars display the SD. $P < 0.05$.

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generated from E17.5 embryos harvested from intercrosses of Wnt5a-heterozygous mice. Analysis of thymocyte populations was performed by staining of the cells after 4 d in culture using antibodies to a panel of lineage-specific cell surface molecules. The results indicate a reduced frequency of DN thymocytes in Wnt5a$^{-/-}$ thymi, but similar percentages and numbers of DP cells produced in Wnt5a$^{-/-}$ and WT control fetal thymi (Fig. 2 A). However, the frequencies and numbers

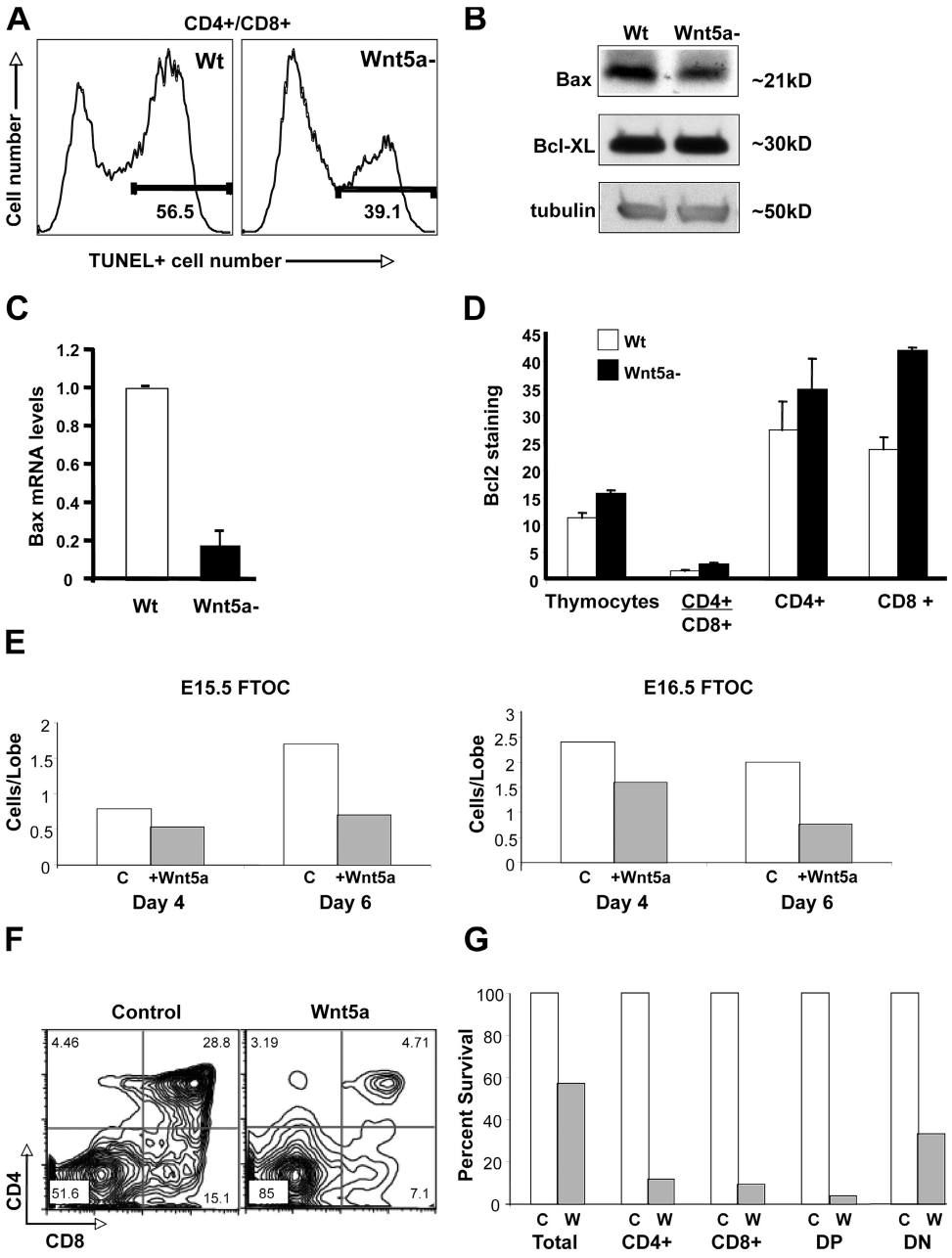

Figure 3. Absence of Wnt5a increases the survival of thymocytes. (A) Representative profiles of TUNEL$^+$ frequencies in DP thymocytes isolated from Wnt5a$^{+/+}$ or Wnt5a$^{-/-}$ FTOCs (E17.5) on day 5 of culture. Bars represent the percentage of TUNEL$^+$ cells. (B) Representative Western blot on ex vivo E18.5 thymocytes with primary antibodies against Bax, Bcl-XL, or tubulin (the loading control). (C) Real-time PCR on total RNA extracted from E18.5 Wnt5a$^{+/+}$ or Wnt5a$^{-/-}$ thymocytes. Bax levels were normalized to Gapdh as an internal control. Results are averaged from three samples per genotype. (D) Bcl2 intercellular staining of thymocyte subsets from E18.5 Wnt5a$^{+/+}$ or Wnt5a$^{-/-}$ thymi. All experiments in A–D were performed three times with similar results. Error bars display the SD. $P < 0.05$. (E) Growth of cells after treatment with Wnt5a in E15.4 (left) or E16.5 (right) FTOCs. Values from eight pooled lobes per sample. This experiment was independently repeated with similar results. (F) Loss of DP and SP cells after Wnt5a treatment. Representative FACS data of three independent experiments is shown. (G) Decreased survival of thymocytes after treatment of FTOCs with Wnt5a. Survival was assayed by FACS analysis on day 4 in culture by counting the numbers of cells not staining for 7-amino-actinomycin-D or Annexin V. The value for untreated cells in each population was set at 100. This experiment was repeated once, with similar qualitative results.

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of $CD4^+$ or $CD8^+$ SP thymocytes were elevated twofold in $Wnt5a^{-/-}$ FTOCs (Fig. 2, A and B). In addition, there was an increase both in the percentage and numbers (Fig. 2 C) of thymocytes expressing high levels of TCR-β receptors, which is consistent with an increase in the development, maintenance, and/or proliferation of mature SP cells in the absence of Wnt5a. No significant difference in the composition and cellularity of γδ T cell lineage was observed in the absence of Wnt5a (unpublished data).

Consistent with the possibility of enhanced growth or survival of αβ T cell lineage, $Wnt5a^{-/-}$ SP $CD4^+$ thymocytes expanded significantly better than WT $CD4^+$ thymocytes when activated by CD3 and CD28 (Fig. 2 D). A previous study showed that canonical Wnt signaling promotes DP cell survival by inducing the antiapoptotic BCL-X_L protein (27). To determine if Wnt5a regulates cell survival, DP cells from day 5 FTOCs were assayed for apoptosis by TUNEL staining (Fig. 3 A). Nearly 60% of the WT DP cells were undergoing apoptosis at this time, whereas <40% of $Wnt5a^{-/-}$ DP cells were apoptotic, suggesting that Wnt5a normally promotes DP cell apoptosis. Western blot analysis of pro- or antiapoptotic proteins in DP cells suggested a reduction in proapoptotic Bax levels in the absence of Wnt5a, whereas Bcl-X_L levels were unchanged (Fig. 3 B). In addition, no differences were detected in the levels of other proapoptotic molecules in these cells, such as the p53-related Puma or Noxa (unpublished data). To confirm the reduction in Bax levels in cells lacking Wnt5a, and to determine if this reduction was occurring at the transcription level, quantitative real-time PCR was performed to compare the levels of Bax mRNA. Compared with WT DP cells, Bax RNA amounts were reduced by 80% in the $Wnt5a^{-/-}$ DP cells (Fig. 3 C), whereas Bcl-X_L levels were unperturbed (not depicted). Furthermore, intracellular staining for the prosurvival molecule Bcl-2 showed upregulation of Bcl2 levels, most notably in $Wnt5a^{-/-}$ $CD8^+$ SP thymocytes. These results indicate diminished apoptosis in thymocytes developing in the absence of Wnt5a, and suggest that Wnt5a normally promotes apoptosis in immature and mature T cells by controlling the relative amounts of proapoptotic Bax versus antiapoptotic Bcl2. To confirm that Wnt5a regulates the survival of thymocytes, we generated FTOCs from WT mice and added exogenous Wnt5a ligand to the culture media. Addition of exogenous Wnt5a inhibited the growth of cells in FTOCs prepared from E15.5 or E16.5 WT embryos (Fig. 3 E, left and right). FTOCs treated with exogenous Wnt5a ligand had less DP and SP cells present on day 4 of culture (Fig. 3 F), and staining of FTOCs with Annexin V and 7-amino-actinomycin-D revealed that addition of exogenous Wnt5a ligand promoted the apoptosis of immature and mature SP thymocytes (Fig. 3 G). In keeping with the findings of reduced Bax mRNA in $Wnt5a^{-/-}$ FTOCs, the addition of exogenous Wnt5a to WT FTOCs moderately increased Bax mRNA levels by 20% (unpublished data).

The results of the FTOC experiments indicate that Wnt5a regulates survival of thymocytes. To demonstrate that the effects were attributable to direct actions of Wnt5a on thymocytes and to examine activation of the noncanonical Wnt

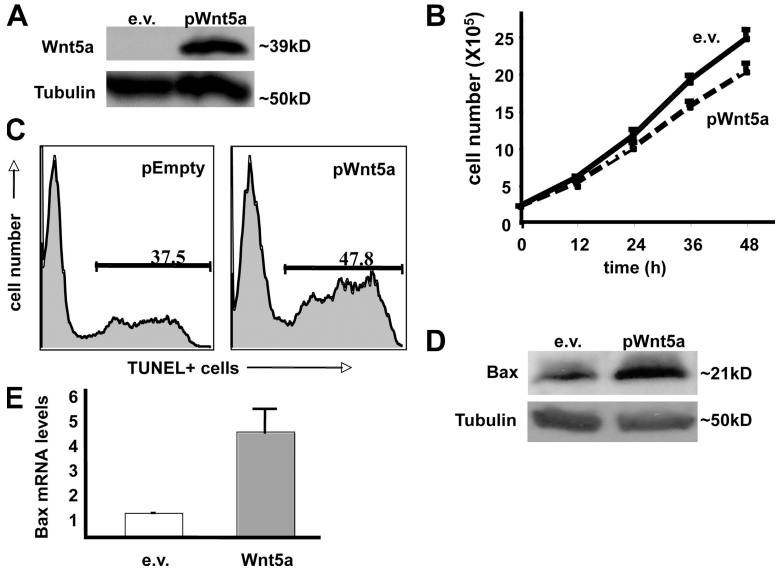

Figure 4. Wnt5a suppresses cell proliferation and promotes apoptosis in a Wnt5a-transduced mouse thymoma cell line. (A) Western blot with primary antibody against mouse Wnt5a on Wnt5a-infected NFC cell extract to verify Wnt5a expression after transduction with the Wnt5a retroviral vector. (B) Proliferation curve of Wnt5a-infected or empty vector–infected (e.v.) NFC cells. Points represent the mean of the samples. Error bars display the SD. (C) TUNEL assay on Wnt5a-infected or control e.v. NFC cells 24 h after serum starvation. Percentage of $TUNEL^+$ cells is given. (D) Western blot on protein extracts of Wnt5a-infected or e.v. NFC cells using primary antibodies against Bax or α-tubulin. (E) Real-time PCR on total RNA extracted from Wnt5a-infected NFC cells. Bax levels were normalized to Gapdh as an internal control. Error bar displays the SD. $P < 0.05$, except $CD4^+$ staining for Bcl2. All experiments were repeated twice, with similar qualitative results.

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signaling pathway in T cells, we ectopically expressed Wnt5a (28) in NFC cells (31), a $CD4^+CD8^+$ αβ lineage T cell line that lacks endogenous Wnt5a expression (Fig. 4 A). NFC-WNT5A cells expanded significantly slower in culture than NFC cells transduced with control empty vector (Fig. 4 B). Furthermore, the level of apoptosis after serum withdrawal was elevated in cells expressing Wnt5a relative to the levels of apoptosis in control NFC cells (Fig. 4 C). In keeping with the results observed in the FTOC experiments, transduction of Wnt5a up-regulated Bax protein levels (Fig. 4 D) concomitant with a fourfold increase in Bax gene expression (Fig. 4 E), confirming that Wnt5a is proapoptotic in thymocytes.

To examine if Wnt5a signals via the noncanonical or canonical Wnt pathway in thymocytes, Wnt–Ca^{2+} signaling and β-catenin stabilization were analyzed in Wnt5a-transduced NFC cells. The levels of activated PKC (Fig. 5 A) and CamKII activity (Fig. 5 B) were clearly elevated in Wnt5a-expressing cells. Furthermore, β-catenin levels were not stimulated, but rather were consistently reduced by Wnt5a expression (Fig. 5 A). These data indicate that Wnt5a is signaling through the noncanonical pathway in thymocytes. To confirm these results in vivo, total protein was extracted from E17.5 thymocytes isolated from WT and $Wnt5a^{-/-}$ littermates, and relative amounts of proteins in the Wnt signaling pathway were assayed. Consistent with the in vitro results, the amount of activated PKC was clearly reduced in $Wnt5a^{-/-}$ thymocytes relative to controls (Fig. 5 C). In addition, β-catenin levels were significantly elevated in $Wnt5a^{-/-}$ thymocytes. In addition, CamKII activity was decreased in thymocytes isolated from thymus of $Wnt5a^{-/-}$ embryos (E18). These data indicate that Wnt5a activates the Wnt–Ca^{2+} pathway in developing thymocytes in vivo, and may inhibit canonical Wnt signaling. Because β-catenin is normally expressed at the highest level in DN thymocytes and $Wnt5a^{-/-}$ mice show a marginal reduction in DN thymocytes, the observed increase in β-catenin amounts cannot be attributed to altered thymic subset composition in $Wnt5a^{-/-}$ thymus. As β-catenin/TCF1 signaling has been shown to ensure the survival of DP thymocytes (27), Wnt5a may normally contribute to the regulation of cell survival by countering the antiapoptotic function of β-catenin/TCF1. However, the consequence of this antagonism does not appear to involve the TCF1 target Bcl-X_L, as Bcl-X_L levels appear unchanged when Wnt5a is not present (Fig. 3 B). Instead, Wnt5a may promote apoptosis by up-regulating Bax gene expression. To determine if Wnt5a can induce alterations in β-catenin activity, NFC cells that were mock transfected or transfected with a Wnt5a expression vector were cotransfected with a β-catenin expression vector and TOPFLASH, which is a reporter plasmid–bearing luciferase gene under transcriptional control of a β-catenin/TCF1–responsive promoter. A plasmid encoding *Renilla* luciferase was also cotransfected as a control for transfection efficiency. Expression of the TOPLASH reporter was not detectible in the absence of cotransfected β-catenin in these cells. Thus, the β-catenin/TCF1–responsive promoter is exclusively dependent on exogenous β-catenin activity. Expression of Wnt5a significantly inhibited activation of the promoter after cotransfection with β-catenin, confirming that Wnt5a inhibits the activity of β-catenin/TCF1 in T cells (Fig. 5 E),

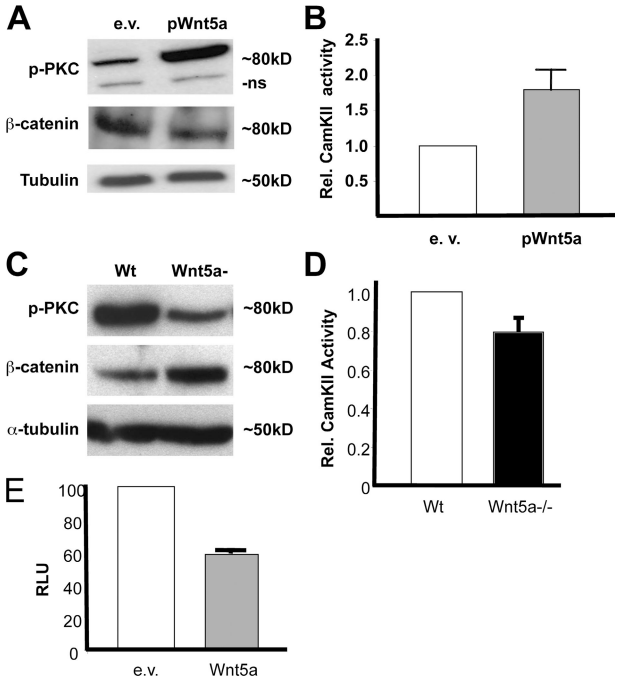

Figure 5. Wnt5a signals through the noncanonical Wnt pathway in thymocytes. Western blot on Wnt5a-infected or empty vector–infected (e.v.) NFC cell protein extracts (A) and on protein extracts of $Wnt5a^{+/+}$ or $Wnt5a^{-/-}$ E18.5 thymocytes (D) with primary antibodies against phosphorylated PKC, β-catenin, or α-tubulin. (B) CamKII activity assays performed on Wnt5a-infected or e.v. NFC cells. (C) Representative Western blot displaying inhibition of activated PKC and β-catenin levels in fetal thymocytes. (D) CamKII assays performed on E18 fetal thymocytes reveals reduced CamKII activity in $Wnt5a^{-/-}$ cells. All experiments in A–D were performed three times with similar results. Error bars represent the SD. $P < 0.05$. (E) Addition of Wnt5a inhibits β-catenin activation of TOPFLASH reporter in NFC cells. RLU, relative luciferase activity. Results are the mean of three independent transfections. This assay was repeated twice, with similar qualitative results. Error bars represent the SD. $P < 0.05$.

The outcome of Wnt5a signaling in hematopoietic cells appears to depend on the cell context. We have previously reported that Wnt5a functions in B cells to inhibit cell proliferation and suppress lymphomagenesis. However, Wnt5a clearly regulates apoptosis in T cells, but not in B cells. And although Wnt5a activates the Wnt–Ca^{2+} pathway in both T and B cells, Wnt5a inhibition of β-catenin was only observed in thymocytes. Lastly, Wnt5a expression is readily detected in B cell precursors, whereas the majority of Wnt5a present in the thymus appears to originate in the stroma. To confirm that Wnt5a produced in the stromal microenvironment could rescue the phenotype of $Wnt5a^{-/-}$ thymocytes, we performed adoptive transplants in $Rag1^{-/-}$ mice using fetal liver cells harvested from E15.5 WT or $Wnt5a^{-/-}$ embryos. No difference was observed in the percentage of DN, DP, or SP

T cells present in thymus or lymph node of mice transplanted with Wnt5a-WT or Wnt5a$^{-/-}$ fetal liver, indicating that Wnt5a in the stromal epithelium of Rag1$^{-/-}$ mice could effectively rescue the development of Wnt5a$^{-/-}$ T cells (unpublished data).

The results of our study using primary fetal Wnt5a$^{-/-}$ cells and a Wnt5a-transduced immature T cell line demonstrate that Wnt5a signaling is necessary for normal T cell development, and that Wnt5a is an inducer of noncanonical Wnt–Ca^{2+} signaling in thymocytes. Our results reveal that noncanonical Wnt signaling promotes cell death and is essential for T cell homeostasis, and that the thymic Wnt signaling is tightly balanced by overall antagonistic cross-regulations mediated by canonical and noncanonical Wnt ligands at distinct stages of thymocyte differentiation.

MATERIALS AND METHODS

FACS. BM cells and thymocytes were isolated from 6–8-wk-old C57BL/6J mice. DN1 (CD44$^+$CD25$^-$) and DN2 (CD44$^+$CD25$^+$) thymocytes, CD4$^+$ and CD8$^+$ thymocytes, and BM-derived pro–/pre–B cells (B220$^+$CD43$^-$ and B220$^+$CD43$^+$ cells) were isolated on a FACSAria cell sorter (BD Biosciences) by FACS.

RNA extraction and RT-PCR. The RNA from sorted cells was isolated using RNeasy Micro kit (QIAGEN), and the RNA equivalent of 10^3 to 5×10^4 cells was reverse transcribed and amplified using SuperSCRIPT III One-Step RT-PCR System (Invitrogen). PCR reactions were performed in a Mastercycler (Eppendorf) for 35 cycles (95°C for 30 s, 57–62°C for 45 s, and 72°C for 30 s) using the following primer sets: Wnt5a (forward, TCGGGACTGGTTGTGGGG; reverse, AGCTCGCAGCCGTCCATC); c-Kit (forward, TCCTCACTCACGGGCGGATC; reverse, TGGAGGTGGGGTGGGGAACT); GATA-3 (forward, GCGGTCCTCAACGGTCAGCAC; reverse, TCGGGCACATAGGGCGGATAG); and HPRT (forward, GTTGAGAGATCATCTCCACC; reverse, AGCGATGATGAACCAGGTTA).

Quantitative real-time PCR. Relative quantities of Bax mRNA were analyzed using a one-step, qRT-PCR reaction performed in the presence of SYBR green (Applied Biosystems) using a real-time PCR machine (DNA Engine Opticon; Bio-Rad Laboratories). The primer sequences (5′ to 3′) were as follows: mouse Bax (forward, CTGAGCTGACCTTGGAGC; reverse, GACTCCAGCCACAAAGATG) and Gapdh control (forward, CACCATGGAGAAGGCCGGGG; reverse, GACGGACACATTGGGGGTAG). The RT-PCR cycling parameters were 48°C for 30 min and 95°C for 10 min, followed by 30 cycles at 94°C for 15 s and 56°C for 1 min. The specificity of amplification was confirmed by agarose gel electrophoresis of the PCR product.

Generation of Wnt5a$^{2/2}$ FTOCs. Mice bearing a mutant Wnt5a allele have been previously described (11). Wnt5a-heterozygous mice were bred to C57BL/6 WT mice for 8 generations to establish the Wnt5a mutation on a (>99.6%) C57BL/6 inbred mouse strain background. FTOCs were derived from E15.5, E16.5, or E17.5 mice, as outlined in the text. Wnt5a$^{-/-}$ embryos and WT embryos were harvested from the same litters, and the genotypes of all samples were confirmed by PCR analysis. All mice were maintained and used in accordance with federal guidelines and the University of Massachusetts Animal Care and Use Committee.

Analysis of thymocytes derived from FTOCs. Thymocytes were harvested from FTOCs on day 4 or 6 of culture, stained with a standard panel mAb specific for T cell lineage antigens, and analyzed using FACSCalibur or sorted using FACSVantage (BD Biosciences). FACS data were processed using FlowJo software (Tree Star, Inc). Proliferation of CD4$^+$ thymocytes was performed by plating 1×10^5 cells into the wells of 96-well plates with 200 μl of RPMI 1640 media supplemented with 20% fetal calf serum in the presence or absence of anti-CD3 and -CD28 antibody-coated beads. After 48 h in culture, the cells were pulsed with [^{3}H]thymidine (1 μCi per well) for 18 h before harvest. Incorporated radioactivity was measured using a liquid scintillation counter (Perkin Elmer Wallace, Inc). Apoptosis of thymocytes was assayed using TUNEL (Guava Technologies, Inc.) and FACS analysis (BD Biosciences), and intracellular BCL-2 staining was performed using anti-Bcl2 mAb (BD Biosciences), according to the manufacturer's protocol. Recombinant mouse Wnt5a ligand (R&D Systems) was used at 400 or 600 ng/ml in the FTOC cultures in the presence of 2 ng/ml of rIL-7 (R&D Systems).

Transduction of Wnt5a in T cells. NFC cells were maintained in complete RPMI media with 10% fetal calf serum. Recombinant retrovirus carrying mouse Wnt5a cDNA was previously described (29). Retroviral-transduced cells expressing GFP were plated in triplicate at 2×10^5 cells per 10-cm plate, and plates were harvested and counted every 12 h to measure the rates of cell proliferation. Transfections of NFC cell were performed using an electroporator (Bio-Rad Laboratories), and a *Renilla* luciferase plasmid (pRL-TK) was added in equal amounts to all transfections. Luciferase data from each sample was first normalized to the *Renilla* luciferase activity to account for differences in transfection efficiencies for each sample.

Western blot analysis. Cells and tissue were lysed with 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor in PBS. Proteins were separated on a 10% polyacrylamide gel and transferred to nitrocellulose membrane. Immunoblots were probed with primary antibodies to Wnt5a (R&D Systems), Bax (BD Biosciences), Bcl-X_L (Cell Signaling Technology), CamKII, CyclinD1 or Cyclin D3 (Santa Cruz Biotechnology, Inc.), phosphorylated PKC-pan (Cell Signaling Technology), or β-catenin-Exon 3 (Calbiochem), and subsequently stained with anti–IgG-HRP antibodies and visualized using ECL (GE Healthcare). An antibody against α-tubulin was used as a loading control (Sigma-Aldrich).

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